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Clostridium thermopalmarium sp. nov., a Moderately Thermophilic Butyrate-Producing Bacterium Isolated from Palm Wine in Senegal

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Summary

A new thermophilic species of the genus *Clostridium* is described, which was isolated from palm wine in Senegal. The cells were anaerobic sporeforming rods, 0.7–1.0 µm wide and 2.0 to 8.0 µm long. The optimum growth temperature was about 55 °C. Sugars were mainly fermented to butyric acid. H_2, CO_2, small amounts of acetate, lactate and ethanol were produced. The G+C content of DNA was 35.7±0.3 mol%. The name *Clostridium thermopalmarium* sp. nov. is proposed for this species; the type strain is DSM 5974.

Key words: *Clostridium thermopalmarium* sp. nov. – Thermophilic – Anaerobic – Butyrate – Palm wine

Introduction

Among mesophilic species of the genus *Clostridium*, several strains produce butyric acid as the main product of sugar metabolism (*Cato* et al., 1986). Recently, *Wiegel* et al. (1989) reported on the isolation of a new moderately thermophilic *Clostridium* species (*Clostridium thermobutyricum*) that produced butyric acid as the major fermentation product suggesting that it was a probable contaminant of *Clostridium thermocellum* strains. Although it has been attempted to isolate the organism from numerous ecosystems, it has been found only in horse manure, associated with the metabolism of cellulose.

Experiments were performed in Senegal to determine wether thermophilic anaerobic bacteria are to be found in palm wine, with a view to searching for ethanol producing microorganisms. Media containing glucose were inoculated with palm wines from various palm groves in Senegal: only two strains grew under moderately thermophilic conditions, and yielded pure cultures. One, a heterolactic amylolytic bacterium, is being studied in our laboratory. Here we report on the other isolate, the sporeforming butyrate producing strain BVP. This organism closely resembles *Clostridium thermobutyricum*. However, on the basis of the differences in metabolic activity, we propose strain BVP as a new species, *Clostridium thermopalmarium* sp. nov.

Materials and Methods

Origin of strain and isolation. Strain BVP was isolated from a palm wine originating from Casamance (southern Senegal). Several samples from various palm groves in northern and southern Senegal were inoculated into a complex rich medium containing glucose as energy source, and incubated at 55 °C. Growth was positive with only one sample from Casamance. A pure culture was obtained using anaerobic techniques (*Hungate*, 1969).

Media. Strain BVP was grown on a medium containing K_2HPO_4, 2.0 g; $MgCl_2 \cdot 6H_2O$, 1.0 g; $MnCl_2 \cdot 2H_2O$, 0.3 g; $(NH_4)_2SO_4$, 3.0 g; cysteine. HCl, 0.5 g; sodium acetate, 0.5 g; yeast extract (Difco, Detroit, MI, USA), 2.0 g; Biotrypcase (Biomérieux, Craponne, France), 2 g; resazurine, 0.001 g; glucose, 10.0 g; mineral solution (*Balch* et al., 1979), 50 ml; trace mineral solution (*Balch* et al., 1979), 5 ml; distilled water, 1 l. The medium was adjusted to pH 7.0 with KOH 10 mol/l, then boiled under a stream of O_2-free N_2 and cooled to room temperature. 20 ml of medium were distributed into 60 ml serum bottles using Hun-

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gate's anaerobic technique (*Hungate*, 1969). Bottles were outgassed with N_2–CO_2 (80–20%) and autoclaved (110 °C for 40 min). After autoclaving, 0.2 ml of 2% $Na_2S \cdot 9H_2O$ and 1 ml of 10% $NaHCO_3$ (sterile, anaerobic solutions) were added to the medium before inoculation. Roll tube media were supplemented with 2% agar (Difco, Detroit, MI, USA).

Analytical techniques. Volatile fatty acids and alcohols were analysed as previously described (*Cord-Ruwish* et al., 1986). Lactate was analysed with the lactate fully enzymatic U.V. system (Boehringer, Mannheim, FRG). Sulfide was measured spectrophotometrically as colloidal CuS (*Cord-Ruwisch*, 1985). Bacterial growth was quantified by measuring the optical density at 580 nm.

Deoxyribonucleic acid base composition. The mol% G+C of the DNA was determined at the DSM, Braunschweig, FRG using the method described by *Meshbah* et al. (1989). DNA was isolated using the modified CTAB method (*Murray* and *Thompson*, 1980).

Electron microscopy. Negative staining was performed with uranyl acetate (4% w/v in distilled water). Cells from an exponential growth culture were fixed for 1h in sodium cacodylate buffer (0.07 M, pH 7.3) containing glutaraldehyde (1.2% w/v) and ruthenium red (0.05% w/v). After washing in cacodylate buffer with ruthenium red, the samples were post-fixed in OsO_4 (1% w/v in cacodylate buffer 0.07 M). Embedding was performed in Epon and ultrathin sections were stained with uranyl acetate (2% w/v in ethanol 50% w/v) and then with lead citrate. Micrographs were taken on a JEOL 1200 CX electron microscope.

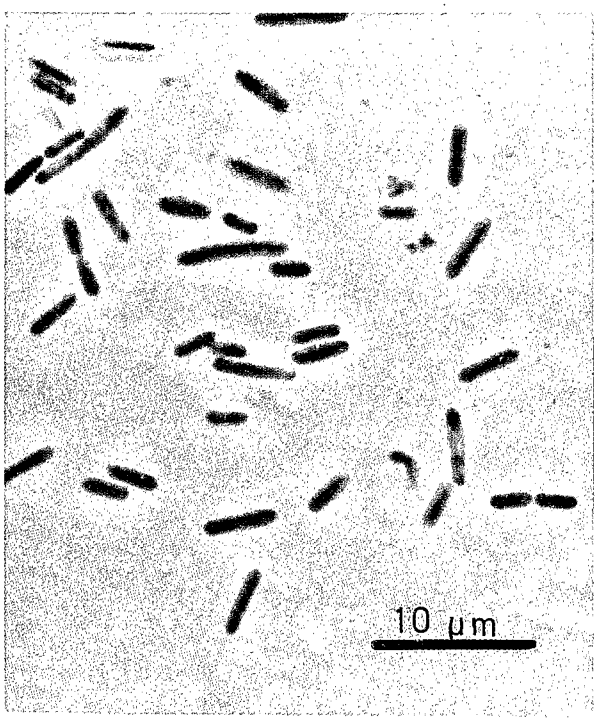

Fig. 1. Phase-contrast photomicrograph of strain BVP grown on glucose.

Results and Discussion

Anaerobic bacterial growth was obtained under thermophilic conditions (50 °C) by inoculating a palm wine sample from Casamance (South Senegal) into a complex glucose enriched medium. After several transfers into the same liquid medium, the enriched culture was immediately diluted in agar tubes. White to creamish colonies were obtained after a one-day incubation at 55 °C. They had an irregularly circular appearence on agar surfaces. Colonies were picked and rediluted in agar dilution series until pure cultures of the organism were obtained.

The newly isolated strain BVP was an obligately anaerobic sporeforming bacterium with characteristics similar to those of the genus *Clostridium* (*Cato* et al., 1986). Its atypical Gram-positive cell wall profile (Fig. 3) excluded it from the genus *Sporomusa* (*Möller* et al., 1984). The vegetative cells of the strain were straight rods, 0.7–1.0 µm in width and 2.0–8.0 µm in length (Fig. 1). The organism produced a terminal to subterminal elliptical endospore which slightly swelled the cell (Fig. 2). The occurrence of spores was rarely observed in repeatedly transferred cultures in liquid medium. The first transfer from a long-stored inoculum (one year at room teperature) resulted in the formation of considerable amounts of spores and swollen cells with no subsequent spore formation (Fig. 2). The cells stained Gram-negative even in the early growth phase. However, strain BVP possessed a cell wall profile of Gram-positive bacteria and a surface layer (Fig. 3). In older cultures, the cells contained inclusion bodies similar to β-hydroxybutyrate granules (Fig. 4). Little motility was observed under the microscope. Peritrich-

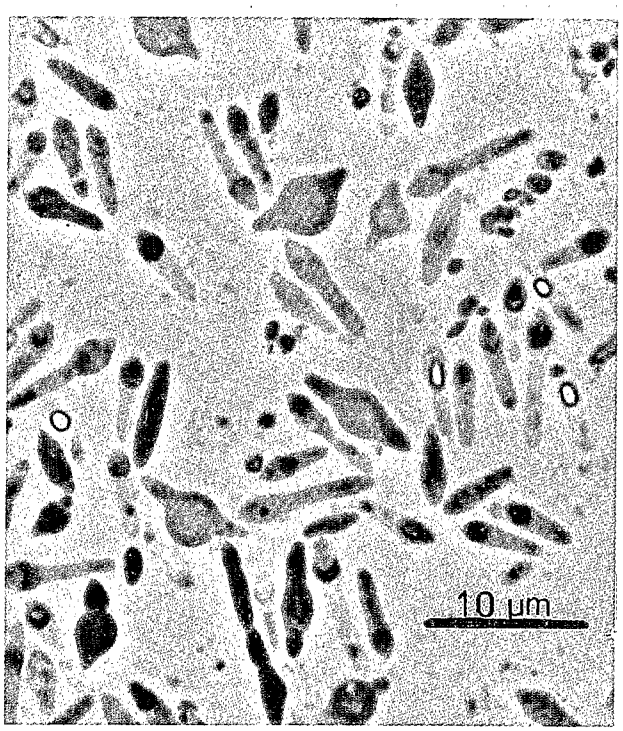

Fig. 2. Phase contrast photomicrograph of strain BVP inoculated into a complex medium obtained with a long-stored inoculum (one year at room temperature). Note the swollen cells.

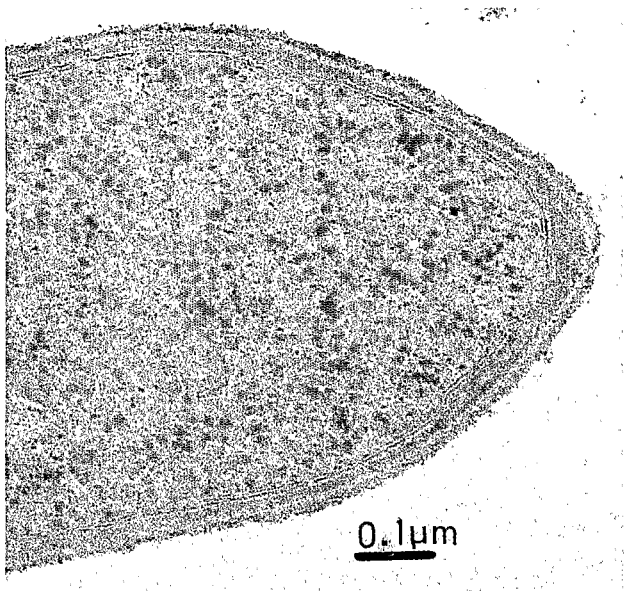

Fig. 3. Transmission electron micrograph of strain BVP showing an atypical Gram-positive cell wall profile.

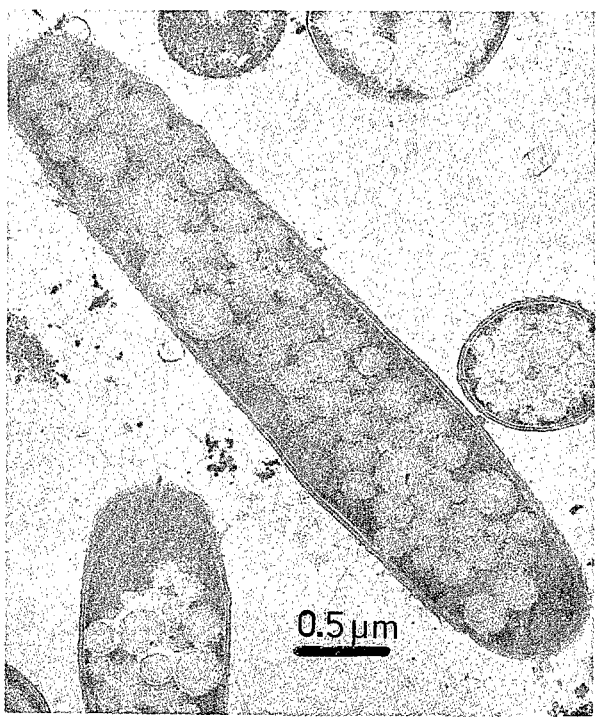

Fig. 4. Transmission electron micrograph of strain BVP. Note the inclusion bodies.

ously disposed flagella were observed using negative cell staining (Fig. 5).

Butyric acid was always the main end product of fermentation (around 1 mol butyric acid produced per mol of glucose consumed). H_2,CO_2, small amounts of acetate, ethanol and lactate were produced. The optimum growth

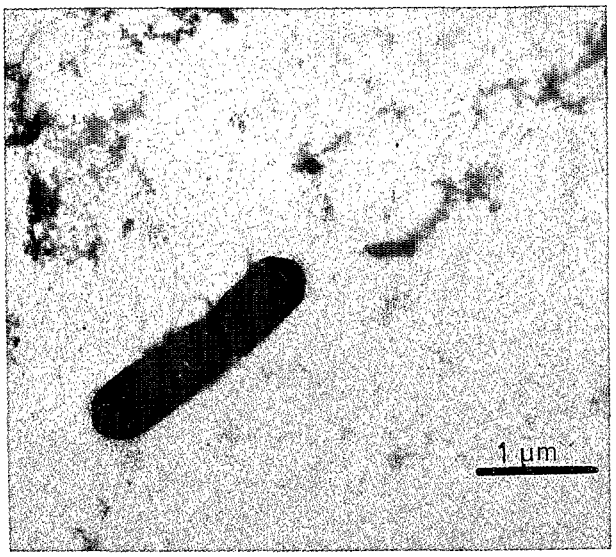

Fig. 5. Transmission electron micrograph of strain BVP. Note the peritrichous flagella.

temperature was 50–55 °C with an upper limit of 60 °C. Thus the organism was moderately thermophilic. The doubling time during growth on sucrose was about 45 min at pH 7.0 and 55 °C. The pH range for growth was pH 6.0 to 8.2, with an optimum at around 6.6 (data not shown).

Strain BVP used various carbohydrates including glucose, xylose, and sucrose. Growth occurred on yeast extract. Yeast extract was necessary for growth and could not be replaced by a vitamin solution (*Pfennig* et al., 1981). Sulfate, thiosulfate and sulfite were not reduced to H_2S.

Five thermophilic species of the genus Clostridium are known to produce butyrate through carbohydrate fermentation (Table 1): *Clostridium thermohydrosulfuricum* (*Wiegel* et al., 1979), *Clostridium thermosaccharolyticum* (*Hollaus* and *Sleytr*, 1972), *Clostridium thermocopriae* (*Jin* et al., 1988), *Clostridium josui* (*Sukhumavasi* et al., 1988), and *Clostridium thermobutyricum* (*Wiegel* et al., 1989). The formation of butyrate by the acetogenic thermophile *Clostridium fervidus* resulted from Trypticase peptone fermentation (*Patel* et al., 1987). However, only *C. thermosaccharolyticum* and *C. thermobutyricum* produced butyric acid as one of the main end products of sugar metabolism. In contrast to *C. thermosaccharolyticum*, strain BVP does not use galactose, lactose and starch, and oxidizes rhamnose and mannitol. Furthermore, it has a higher G+C content of the DNA. Strain BVP differs from *C. thermobutyricum* in the following two important respects: (i) ethanol is produced from glucose fermentation, (ii) it ferments sucrose. Thus we propose that strain BVP be placed in the genus *Clostridium* as a new species, *Clostridium thermopalmarium* sp. nov.

Recently *Worden* et al. (1989) reported on several potential commercial applications of butyrate, including the production of butanol. Thermophiles are known to have higher growth and metabolic rates than mesophiles.

Table 1. Comparison of characteristics of thermophilic *Clostridium* species producing butyrate

Characteristic	*C. fervidus**	*C. josui*	*C. thermocopriae*	*C. thermosaccharolyticum*	*C. thermohydrosulfuricum*	*C. thermobutyricum*	*C. thermopalmarium* sp. nov.
Acid produced from:							
Cellulose	−	+	+	−	−	−	−
Sucrose	−	−	w	+	+	−	+
Growth at 70 °C	+	−	+	−	+	−	−
Ethanol production from carbohydrates	+	+	+	+	+	−	+
G+C content (mol%)	39	40	36.7–36.8	29–32	35–37	37	35.7
Reference	*Patel* et al., 1987	*Sukhumavasi* et al., 1988	*Jin* et al., 1988	*Hollaus* and *Sleytr*, 1972 *Cato* et al., 1986	*Wiegel* et al., 1979 *Cato* et al., 1986	*Wiegel* et al., 1989	this paper

+, positive reaction; −, negative reaction; w, weak reaction depending on strains.
* butyrate is produced from Trypticase peptone medium (*Patel* et al., 1987).

Furthermore, growth at high temperature facilitates the removal of volatile products (*Payton*, 1984). Studies on butyrate production using strain BVP should therefore be of biotechnological interest.

Description of *Clostridium thermopalmarium* spec. nov. (*ther. mo. pal. ma'ri. um.* Gr. adj. thermos, hot; L. adj. *palmarius*, of palm tree; L. neut. aldj. *thermoplamarium*, referring to its thermophily and isolation from palm wine.

The cells are straight rods, 0.7–1.0 μm wide and 2.0–8.0 μm long. The organism produces subterminal to terminal endospores with slightly swollen sporangia. Strictly anaerobic. The optimum growth temperature is between 50 and 55 °C; upper limit of growth at 60 °C; the optimum pH is about 6.6; ferments glucose, fructose, maltose, pyruvate, xylose, ribose, rahmnose, mannitol, sucrose, cellobiose. Not used: H_2/CO_2, acetate, propionate, butyrate, lactate, lactose, sorbose, melibiose, galactose, arabinose, amylose, glycerol, adonitol, dulcitol, methanol, starch, cellulose, Biotrypcase. In the fermentation of carbohydrates, butyric acid is one of the main end products. H_2, CO_2, small amounts of acetate, lactate and ethanol are produced from glucose fermentation. Yeast extract is necessary for growth.

The G+C mol% of the DNA is 35.7±0.3.

Isolated from palm wine in Senegal.

The type strain is strain BVP (DSM 5974).

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